ANTISENSE MODULATION OF HORMONE-SENSITIVE LIPASE EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods
5 for modulating the expression of hormone-sensitive lipase.
In particular, this invention relates to compounds,
particularly oligonucleotides, specifically hybridizable with
nucleic acids encoding hormone-sensitive lipase. Such
compounds have been shown to modulate the expression of
10 hormone-sensitive lipase.

BACKGROUND OF THE INVENTION

The mobilization of fatty acid from triglycerides and
cholesterol esters provides the primary source of energy in
mammals. Hormone-sensitive lipase (also known as HSL, LIPE and
15 neutral cholesterol ester hydrolase; NCEH) is a
multifunctional tissue lipase that plays a critical role in
this process. The enzyme has broad specificity, catalyzing the
hydrolysis of tri-, di-, and monoacylglycerols, as well as
cholesterol esters. Hormone-sensitive lipase has been studied
20 most extensively in adipose tissue, where it is thought to
catalyze the major rate-limiting step in lipolysis (Saltiel,
Proc. Natl. Acad. Sci. U S A, **2000**, *97*, 535-537).

Hormone-sensitive lipase is acutely activated by cAMP-
dependent phosphorylation and its regulation in adipocytes is
25 the primary means by which lipolytic agents, such as
catecholamines, control the circulating levels of free fatty
acids.

Free fatty acids in the plasma profoundly influence
carbohydrate and lipid utilization, storage, and synthesis,

both in liver and muscle. Products of fatty acid metabolism are also thought to bind directly to nuclear receptors, thus regulating transcription of genes involved in lipid synthesis and breakdown. These observations suggest that hormone-
5 sensitive lipase is an important player in controlling the balance of substrate utilization and storage (Saltiel, *Proc. Natl. Acad. Sci. U S A,* **2000,** *97,* 535-537).

In addition to adipocytes, hormone-sensitive lipase is expressed in skeletal muscle, heart, brain, pancreatic beta
10 cells, adrenal gland, ovaries, testes, and macrophages. Although triglyceride hydrolysis is also important in muscle and pancreas, cholesterol ester hydrolysis appears to play a separate biological role in these tissues (Saltiel, *Proc. Natl. Acad. Sci. U S A,* **2000,** *97,* 535-537).

15 The human hormone-sensitive lipase gene was cloned in 1988 (Holm et al., *Science,* **1988,** *241,* 1503-1506) and mapped to chromosome 19q13.1-13.2 (Levitt et al., *Cytogenet. Cell Genet.,* **1995,** *69,* 211-214).

The size of hormone-sensitive lipase gene products is
20 variable. In rat, the heart, skeletal muscle, placenta and ovaries express slightly larger mRNAs (3.5 kb) than the mRNAs expressed in adipose tissue (3.3 kb). In addition, a 3.9 kb mRNA is expressed in testis (HSL_{tes})(Holm et al., *Science,* **1988,** *241,* 1503-1506; Holst et al., *Genomics,* **1996,** *35,* 441-
25 447) as a distinct isoform (Holst et al., *Genomics,* **1996,** *35,* 441-447).

Macrophage-specific overexpression of hormone-sensitive lipase in transgenic mice has indicated a greater susceptibility for development of atherosclerosis (Escary et
30 al., *J. Lipid Res.,* **1999,** *40,* 397-404).

Targeted disruption of the gene in transgenic mice has further shown that hormone-sensitive lipase is required for spermatogenesis but is not the only enzyme involved in mediation of hydrolysis of triacylglycerol stored in

adipocytes (Osuga et al., *Proc. Natl. Acad. Sci. U S A*, **2000**, *97*, 787-792).

It has been demonstrated that diabetic patients are at increased risk to develop atherosclerotic vascular disease. Support for this conclusion can be found in studies of rat and mouse beta cells wherein hormone-sensitive lipase activation via lipid-derived signals, contributes to the overall release of insulin. This release may adversely affect beta-cells in events leading to non-insulin dependent diabetes mellitus (NIDDM), where hyperglycemia is accompanied by abnormalities in lipid metabolism (Mulder et al., *Diabetes*, **1999**, *48*, 228-232).

Results from study of ovarian cancer patients have demonstrated increased levels of hormone-sensitive lipase in normal adipocytes and suggest a critical role for hormone-sensitive lipase in cancer-mediated defects of lipid metabolism (Gercel-Taylor et al., *Gynecol. Oncol.*, **1996**, *60*, 35-41).

A defect in hormone-sensitive lipase has been demonstrated to confer resistance to catecholamine-induced lipolysis which leads to an adipocyte abnormality associated with familial obesity (Hellstrom et al., *Diabetologia*, **1996**, *39*, 921-928).

Several inhibitors of hormone-sensitive lipase have been described in the art. These include antibodies, small molecules, and antisense nucleic acids.

Small molecule inhibitors of hormone-sensitive lipase have been disclosed and claimed in PCT publications WO 01/17981 (Petry et al., **2001**), WO 00/67025 (Mueller et al., **2000**), and WO 00/27388 (Wagle et al., **2000**).

Tolbutamide was demonstrated to reduce the activity of hormone-sensitive lipase in rat adipocytes *in vitro*. However, treatment of type 2 diabetic patients with tolbutamide showed

no benefit compared to placebo-treated patients (Agardh et al., *Diabetes Res. Clin. Pract.*, **1999**, *46*, 99-108).

Disclosed and claimed in PCT publication WO 01/26664 is the use of an antisense inhibitor to inhibit fertility in a

5 male animal wherein said antisense inhibitor is substantially complementary to a portion of an mRNA encoding hormone-sensitive lipase and wherein said inhibitor comprises at least five contiguous bases (Mitchell and Wang, **2001**).

A vector containing a 387-nucleotide fragment of rat

10 hormone-sensitive lipase in the antisense direction was used in investigations of the role of the hormone-sensitive lipase gene in the activity of neutral cholesterol ester hydrolase in Chinese hamster ovary (CHO) cells and concluded that, in fact, hormone-sensitive lipase and neutral cholesterol ester

15 hydrolase are the same enzyme in macrophages (Osuga et al., *Biochem. Biophys. Res. Commun.*, **1997**, *233*, 655-657).

The involvement of hormone-sensitive lipase in disorders caused by aberrant lipid metabolism make it a potentially useful therapeutic target for intervention in conditions such

20 as obesity, diabetes and atherosclerotic vascular disease.

Currently, inhibitors of hormone-sensitive lipase include natural metabolites (Jepson and Yeaman, *FEBS Lett.*, **1992**, *310*, 197-200; Plee-Gautier et al., *Biochem. J.*, **1996**, *318*, 1057-1063) and the previously cited small molecules,

25 antibodies and antisense inhibitors. There remains, however, a long felt need for additional agents capable of effectively and selectively inhibiting the function of hormone-sensitive lipase.

Antisense technology is emerging as an effective means

30 for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of expression of hormone-sensitive lipase.

The present invention provides compositions and methods
for modulating expression of hormone-sensitive lipase,
including modulation of isoforms of hormone-sensitive lipase,
including the testis-specific hormone-sensitive lipase known
5 as HSL_{tes}.

SUMMARY OF THE INVENTION

The present invention is directed to compounds,
particularly antisense oligonucleotides, which are targeted
to a nucleic acid encoding hormone-sensitive lipase, and which
10 modulate the expression of hormone-sensitive lipase.
Pharmaceutical and other compositions comprising the compounds
of the invention are also provided. Further provided are
methods of modulating the expression of hormone-sensitive
lipase in cells or tissues comprising contacting said cells
15 or tissues with one or more of the antisense compounds or
compositions of the invention. Further provided are methods
of treating an animal, particularly a human, suspected of
having or being prone to a disease or condition associated
with expression of hormone-sensitive lipase by administering
20 a therapeutically or prophylactically effective amount of one
or more of the antisense compounds or compositions of the
invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds,
25 particularly antisense oligonucleotides, for use in modulating
the function of nucleic acid molecules encoding hormone-
sensitive lipase, ultimately modulating the amount of hormone-
sensitive lipase produced. This is accomplished by providing
antisense compounds which specifically hybridize with one or
30 more nucleic acids encoding hormone-sensitive lipase. As used
herein, the terms "target nucleic acid" and "nucleic acid
encoding hormone-sensitive lipase" encompass DNA encoding
hormone-sensitive lipase, RNA (including pre-mRNA and mRNA)

transcribed from such DNA, and also cDNA derived from such
RNA.   The specific hybridization of an oligomeric compound
with its target nucleic acid interferes with the normal
function of the nucleic acid. This modulation of function of

5   a target nucleic acid by compounds which specifically
hybridize to it is generally referred to as "antisense". The
functions of DNA to be interfered with include replication and
transcription.   The functions of RNA to be interfered with
include all vital functions such as, for example,

10 translocation of the RNA to the site of protein translation,
translation of protein from the RNA, splicing of the RNA to
yield one or more mRNA species, and catalytic activity which
may be engaged in or facilitated by the RNA.   The overall
effect of such interference with target nucleic acid function

15 is modulation of the expression of hormone-sensitive lipase.
In the context of the present invention, "modulation" means
either an increase (stimulation) or a decrease (inhibition)
in the expression of a gene.   In the context of the present
invention, inhibition is the preferred form of modulation of

20 gene expression and mRNA is a preferred target.

        It is preferred to target specific nucleic acids for
antisense.   "Targeting" an antisense compound to a particular
nucleic acid, in the context of this invention, is a multistep
process.   The process usually begins with the identification

25 of a nucleic acid sequence whose function is to be modulated.
This may be, for example, a cellular gene (or mRNA transcribed
from the gene) whose expression is associated with a
particular disorder or disease state, or a nucleic acid
molecule from an infectious agent.   In the present invention,

30 the target is a nucleic acid molecule encoding hormone-
sensitive lipase.   The targeting process also includes
determination of a site or sites within this gene for the
antisense interaction to occur such that the desired effect,
e.g., detection or modulation of expression of the protein,

35 will result. Within the context of the present invention, a

preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in

5   transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and

10 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is

15 also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the

20 invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding hormone-sensitive lipase, regardless of the sequence(s) of such codons.

25   It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and

30 "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region"

35 refer to a portion of such an mRNA or gene that encompasses

from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which
5 is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA
10 in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA
15 in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue
20 of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

25   Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a
30 continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease.
35 Aberrant fusion junctions due to rearrangements or deletions

are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

5   Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity

to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in

5 the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of

10 these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention

15 encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of

20 ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

25 For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire

30 complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene

35 expression as they pertain, for example, to disease

association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other

5 compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression)(Madden,

10 et al., *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays

15 and proteomics (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, et al., *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et

20 al., *Anal. Biochem.*, **2000**, 286, 91-98; Larson, et al., *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**,

25 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**, 35, 1895-904) and mass spectrometry methods (reviewed in (To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The specificity and sensitivity of antisense is also

30 harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and

numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals,

5 especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides

10 composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because

15 of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other

20 oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e., from about 8 to about 50 linked nucleosides).

25 Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic

30 oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such

35 heterocyclic bases are the purines and the pyrimidines.

Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to

5 either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular

10 structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5'

15 phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having

20 modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside

25 backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri- esters, methyl and other alkyl phosphonates including 3'-

30 alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'- amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-

35 phosphates having normal 3'-5' linkages, 2'-5' linked analogs

of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most

5 internucleotide linkage, i.e., a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the

10 preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925;

15 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

20   Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain

25 heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and

30 thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141;   5,235,033;   5,264,562;   5,264,564;   5,405,938;
5   5,434,257;   5,466,677;   5,470,967;   5,489,677;   5,541,307; 5,561,225;   5,596,086;   5,602,240;   5,610,289;   5,602,240; 5,608,046;   5,610,289;   5,618,704;   5,623,070;   5,663,312; 5,633,360;   5,677,437;   5,792,608;   5,646,269   and   5,677,439, certain of which are commonly owned with this application, and
10 each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate
15 nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide
20 containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited
25 to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are
30 oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known   as   a   methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-$ $N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein   the   native
35 phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the

above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

5   Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may

10 be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_2CH_3$, $O(CH_2)_nONH_2$ and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the

15 following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino,

20 substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred

25 modification includes 2'-methoxyethoxy ($2'-O-CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also

30 known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., $2'-O-CH_2-O-CH_2-N(CH_2)_2$, also described in examples hereinbelow.‐

      A further prefered modification includes Locked Nucleic

35 Acids (LNAs) in which the 2'-hydroxyl group is linked to the

3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne ($-CH_2-$)$_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are

5   described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-OCH CH CH NH), 2'-allyl (2'-CH - $_2$ CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or

10   ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal

15   nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080;

20   5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which

25   is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine

30   (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine

35   and guanine, 2-propyl and other alkyl derivatives of adenine

and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil),

5 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and

10 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-

15 clamps such as a substituted phenoxazine cytidine (e.g., 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include

20 those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of*

25 *Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke,

30 S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-

adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and* 5 *Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the 10 preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 15 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is 20 commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, 25 cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, 30 polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, 35 folate, phenanthridine, anthraquinone, acridine, fluoresceins,

rhodamines, coumarins, and dyes. Groups that enhance the
pharmacodynamic properties, in the context of this invention,
include groups that improve oligomer uptake, enhance oligomer
resistance to degradation, and/or strengthen sequence-specific
5 hybridization with RNA. Groups that enhance the pharmaco-
kinetic properties, in the context of this invention, include
groups that improve oligomer uptake, distribution, metabolism
or excretion. Representative conjugate groups are disclosed
in International Patent Application PCT/US92/09196, filed
10 October 23, 1992 the entire disclosure of which is incor-
porated herein by reference. Conjugate moieties include but
are not limited to lipid moieties such as a cholesterol moiety
(Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-
6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*,
15 **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol
(Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309;
Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-
2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*,
1992, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or
20 undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*,
1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330;
Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a
phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-
ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate
25 (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654;
Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a
polyamine or a polyethylene glycol chain (Manoharan et al.,
Nucleosides & Nucleotides, **1995**, *14*, 969-973), or adamantane
acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*,
30 3651-3654), a palmityl moiety (Mishra et al., *Biochim.
Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or
hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J.
Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937. Oligonucleotides
of the invention may also be conjugated to active drug

substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide,

5 chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein

10 by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717,

15 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;

20 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and

25 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one

30 of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this

35 invention, are antisense compounds, particularly

oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region

5   wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for

10 enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide

15 inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely

20 detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

        Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides

25 and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775;

30 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

        The antisense compounds used in accordance with this

35 invention may be conveniently and routinely made through the

well-known technique of solid phase synthesis. Equipment for
such synthesis is sold by several vendors including, for
example, Applied Biosystems (Foster City, CA). Any other
means for such synthesis known in the art may additionally or
5   alternatively be employed. It is well known to use similar
techniques   to   prepare   oligonucleotides   such   as   the
phosphorothioates and alkylated derivatives.

        The antisense compounds of the invention are synthesized
in vitro and do not include antisense compositions of
10 biological origin, or genetic vector constructs designed to
direct the in vivo synthesis of antisense molecules.
The   compounds   of   the   invention   may   also   be   admixed,
encapsulated, conjugated or otherwise associated with other
molecules, molecule structures or mixtures of compounds, as
15 for example, liposomes, receptor targeted molecules, oral,
rectal,   topical   or   other   formulations,   for   assisting   in
uptake, distribution and/or absorption. Representative United
States patents that teach the preparation of such uptake,
distribution and/or absorption assisting formulations include,
20 but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016;
    5,459,127;   5,521,291;   5,543,158;   5,547,932;   5,583,020;
    5,591,721;   4,426,330;   4,534,899;   5,013,556;   5,108,921;
    5,213,804;   5,227,170;   5,264,221;   5,356,633;   5,395,619;
    5,416,016;   5,417,978;   5,462,854;   5,469,854;   5,512,295;
25 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and
    5,595,756, each of which is herein incorporated by reference.
        The antisense compounds of the invention encompass any
pharmaceutically acceptable salts, esters, or salts of such
esters, or any other compound which, upon administration to
30 an animal including a human, is capable of providing (directly
or indirectly) the biologically active metabolite or residue
thereof. Accordingly, for example, the disclosure is also
drawn to prodrugs and pharmaceutically acceptable salts of the
compounds of the invention, pharmaceutically acceptable salts
35 of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or

5 conditions.   In   particular,   prodrug   versions   of   the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S.

10 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not

15 impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the

20 like.   Examples   of   suitable   amines   are N,N'-dibenzylethylenediamine,   chloroprocaine,   choline, diethanolamine,   dicyclohexylamine,   ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-

25 19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the

30 free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein,

35 a "pharmaceutical addition salt" includes a pharmaceutically

acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and

5   phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or

10   phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid,

15   gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved

20   in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid,

25   naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be

30   prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

35   For oligonucleotides, preferred examples of

pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with

5   inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid,

10 malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from

15 elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder

20 which can be treated by modulating the expression of hormone-sensitive lipase is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense

25 compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

30 The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding hormone-sensitive lipase, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides

35 of the invention with a nucleic acid encoding hormone-

sensitive lipase can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection

5 means for detecting the level of hormone-sensitive lipase in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions

10 of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by

15 inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or

20 intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical

25 administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also

30 be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral

35 (e.g., dioleoylphosphatidyl DOPE ethanolamine,

dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl
choline) negative (e.g., dimyristoylphosphatidyl glycerol
DMPG) and cationic (e.g., dioleoyltetramethylaminopropyl DOTAP
and   dioleoylphosphatidyl   ethanolamine   DOTMA).

5 Oligonucleotides of the invention may be encapsulated within
liposomes or may form complexes thereto, in particular to
cationic liposomes. Alternatively, oligonucleotides may be
complexed to lipids, in particular to cationic lipids.
Preferred fatty acids and esters include but are not limited

10 arachidonic acid, oleic acid, eicosanoic acid, lauric acid,
caprylic acid, capric acid, myristic acid, palmitic acid,
stearic acid, linoleic acid, linolenic acid, dicaprate,
tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate,
1-dodecylazacycloheptan-2-one,   an   acylcarnitine,   an

15 acylcholine, or a C_{1-10} alkyl ester (e.g., isopropylmyristate
IPM),   monoglyceride,   diglyceride   or   pharmaceutically
acceptable salt thereof. Topical formulations are described
in detail in United States patent application 09/315,298 filed
on May 20, 1999 which is incorporated herein by reference in

20 its entirety.

Compositions and formulations for oral administration
include   powders   or   granules,   microparticulates,
nanoparticulates, suspensions or solutions in water or non-
aqueous media, capsules, gel capsules, sachets, tablets or

25 minitablets.   Thickeners,   flavoring   agents,   diluents,
emulsifiers, dispersing aids or binders may be desirable.
Preferred   oral   formulations   are   those   in   which
oligonucleotides of the invention are administered in
conjunction with one or more penetration enhancers surfactants

30 and chelators.   Preferred surfactants include fatty acids
and/or esters or salts thereof, bile acids and/or salts
thereof. Prefered bile acids/salts include chenodeoxycholic
acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic
acid, dehydrocholic acid, deoxycholic acid, glucholic acid,

35 glycholic acid, glycodeoxycholic acid, taurocholic acid,

taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate,
sodium glycodihydrofusidate.   Prefered fatty acids include
arachidonic acid, undecanoic acid, oleic acid, lauric acid,
caprylic acid, capric acid, myristic acid, palmitic acid,
5   stearic acid, linoleic acid, linolenic acid, dicaprate,
tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-
dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine,
or a monoglyceride, a diglyceride or a pharmaceutically
acceptable salt thereof (e.g., sodium).   Also prefered are
10 combinations of penetration enhancers, for example, fatty
acids/salts in combination with bile acids/salts.   A
particularly prefered combination is the sodium salt of lauric
acid, capric acid and UDCA.   Further penetration enhancers
include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-
15 cetyl ether.   Oligonucleotides of the invention may be
delivered orally in granular form including sprayed dried
particles, or complexed to form micro or nanoparticles.
Oligonucleotide complexing agents include
poly-amino   acids;   polyimines;   polyacrylates;
20 polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates;
cationized   gelatins,   albumins,   starches,   acrylates,
polyethyleneglycols   (PEG)   and   starches;
polyalkylcyanoacrylates;   DEAE-derivatized   polyimines,
pollulans, celluloses and starches.   Particularly preferred
25 complexing agents include chitosan, N-trimethylchitosan, poly-
L-lysine,   polyhistidine,   polyornithine,   polyspermines,
protamine,   polyvinylpyridine,   polythiodiethylamino-
methylethylene P(TDAE), polyaminostyrene (e.g., p-amino),
poly(methylcyanoacrylate),   poly(ethylcyanoacrylate),
30 poly(butylcyanoacrylate),   poly(isobutylcyanoacrylate),
poly(isohexylcynaoacrylate),   DEAE-methacrylate,   DEAE-
hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran,
polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid),
poly(DL-lactic-co-glycolic   acid   (PLGA),   alginate,   and
35 polyethyleneglycol   (PEG).   Oral   formulations   for

oligonucleotides and their preparation are described in detail
in United States applications 08/886,829 (filed July 1, 1997),
09/108,673 (filed July 1, 1998), 09/256,515 (filed February
23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298
5 (filed May 20, 1999) each of which is incorporated herein by
reference in their entirety.

Compositions and formulations for parenteral,
intrathecal or intraventricular administration may include
sterile aqueous solutions which may also contain buffers,
10 diluents and other suitable additives such as, but not limited
to, penetration enhancers, carrier compounds and other
pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention
include, but are not limited to, solutions, emulsions, and
15 liposome-containing formulations. These compositions may be
generated from a variety of components that include, but are
not limited to, preformed liquids, self-emulsifying solids and
self-emulsifying semisolids.

The pharmaceutical formulations of the present
20 invention, which may conveniently be presented in unit dosage
form, may be prepared according to conventional techniques
well known in the pharmaceutical industry. Such techniques
include the step of bringing into association the active
ingredients with the pharmaceutical carrier(s) or
25 excipient(s). In general the formulations are prepared by
uniformly and intimately bringing into association the active
ingredients with liquid carriers or finely divided solid
carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be
30 formulated into any of many possible dosage forms such as, but
not limited to, tablets, capsules, gel capsules, liquid
syrups, soft gels, suppositories, and enemas. The
compositions of the present invention may also be formulated
as suspensions in aqueous, non-aqueous or mixed media.
35 Aqueous suspensions may further contain substances which

increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

5   In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the

10   final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

15   The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and

20   Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker

25   (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with

30   each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a

water-in-oil (w/o) emulsion. Alternatively, when an oily
phase is finely divided into and dispersed as minute droplets
into a bulk aqueous phase the resulting composition is called
an oil-in-water (o/w) emulsion. Emulsions may contain

5 additional components in addition to the dispersed phases and
the active drug which may be present as a solution in either
the aqueous phase, oily phase or itself as a separate phase.
Pharmaceutical excipients such as emulsifiers, stabilizers,
dyes, and anti-oxidants may also be present in emulsions as

10 needed. Pharmaceutical emulsions may also be multiple
emulsions that are comprised of more than two phases such as,
for example, in the case of oil-in-water-in-oil (o/w/o) and
water-in-oil-in-water (w/o/w) emulsions. Such complex
formulations often provide certain advantages that simple

15 binary emulsions do not. Multiple emulsions in which
individual oil droplets of an o/w emulsion enclose small water
droplets constitute a w/o/w emulsion. Likewise a system of
oil droplets enclosed in globules of water stabilized in an
oily continuous provides an o/w/o emulsion.

20   Emulsions are characterized by little or no
thermodynamic stability. Often, the dispersed or
discontinuous phase of the emulsion is well dispersed into the
external or continuous phase and maintained in this form
through the means of emulsifiers or the viscosity of the

25 formulation. Either of the phases of the emulsion may be a
semisolid or a solid, as is the case of emulsion-style
ointment bases and creams. Other means of stabilizing
emulsions entail the use of emulsifiers that may be
incorporated into either phase of the emulsion. Emulsifiers

30 may broadly be classified into four categories: synthetic
surfactants, naturally occurring emulsifiers, absorption
bases, and finely dispersed solids (Idson, in *Pharmaceutical
Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**,
Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker
5   (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic
10 portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different
15 classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

20   Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous
25 lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite,
30 hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the

properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker

5 (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally

10 occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose

15 ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

20   Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben,

25 propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl

30 gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via

35 dermatological, oral and parenteral routes and methods for

their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been

5 very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*,

10 Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

15   In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid

20 solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient

25 amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-

30 active molecules (Leung and Shah, *in: Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant

and electrolyte. Whether the microemulsion is of the water-
in-oil (w/o) or an oil-in-water (o/w) type is dependent on the
properties of the oil and surfactant used and on the structure
and geometric packing of the polar heads and hydrocarbon tails
5 of the surfactant molecules (Schott, in *Remington's*
Pharmaceutical Sciences, Mack Publishing Co., Easton, PA,
1985, p. 271).

     The phenomenological approach utilizing phase diagrams
has been extensively studied and has yielded a comprehensive
10 knowledge, to one skilled in the art, of how to formulate
microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*,
Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,
Inc., New York, N.Y., volume 1, p. 245; Block, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker
15 (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1,
p. 335). Compared to conventional emulsions, microemulsions
offer the advantage of solubilizing water-insoluble drugs in
a formulation of thermodynamically stable droplets that are
formed spontaneously.
20   Surfactants used in the preparation of microemulsions
include, but are not limited to, ionic surfactants, non-ionic
surfactants, Brij 96, polyoxyethylene oleyl ethers,
polyglycerol fatty acid esters, tetraglycerol monolaurate
(ML310), tetraglycerol monooleate (MO310), hexaglycerol
25 monooleate (PO310), hexaglycerol pentaoleate (PO500),
decaglycerol monocaprate (MCA750), decaglycerol monooleate
(MO750), decaglycerol sequioleate (SO750), decaglycerol
decaoleate (DAO750), alone or in combination with
cosurfactants.   The cosurfactant, usually a short-chain
30 alcohol such as ethanol, 1-propanol, and 1-butanol, serves to
increase the interfacial fluidity by penetrating into the
surfactant film and consequently creating a disordered film
because of the void space generated among surfactant
molecules. Microemulsions may, however, be prepared without

the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols,

5 propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols,

10 polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have

15 been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug

20 from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al.,

25 *Pharmaceutical Research*, **1994**, *11*, 1385; Ho et al., *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or

30 oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption

of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas

5 of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance

10 the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-

15 surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides

20 microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug

25 delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and

30 an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which
5   is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated
10   drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are
15   the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological
20   membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

25   Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high
30   systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes
35   to deliver agents including high-molecular weight DNA into the

skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

5   Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an

10 endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, *1987, 147*, 980-985).

          Liposomes which are pH-sensitive or negatively-charged,

15 entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine

20 kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, *1992, 19*, 269-274).

          One major type of liposomal composition includes phospholipids other than naturally-derived

25 phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are

30 formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of
liposomal drug formulations to the skin. Application of
liposomes containing interferon to guinea pig skin resulted
in a reduction of skin herpes sores while delivery of
5 interferon via other means (e.g., as a solution or as an
emulsion) were ineffective (Weiner et al., *Journal of Drug
Targeting*, **1992**, *2*, 405-410). Further, an additional study
tested the efficacy of interferon administered as part of a
liposomal formulation to the administration of interferon
10 using an aqueous system, and concluded that the liposomal
formulation was superior to aqueous administration (du Plessis
et al., *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to
determine their utility in the delivery of drugs to the skin,
15 in particular systems comprising non-ionic surfactant and
cholesterol. Non-ionic liposomal formulations comprising
Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-
10-stearyl ether) and Novasome™ II (glyceryl distearate/
cholesterol/polyoxyethylene-10-stearyl ether) were used to
20 deliver cyclosporin-A into the dermis of mouse skin. Results
indicated that such non-ionic liposomal systems were effective
in facilitating the deposition of cyclosporin-A into different
layers of the skin (Hu et al., *S.T.P.Pharma. Sci.*, **1994**, *4*,
6, 466).

25 Liposomes also include "sterically stabilized"
liposomes, a term which, as used herein, refers to liposomes
comprising one or more specialized lipids that, when
incorporated into liposomes, result in enhanced circulation
lifetimes relative to liposomes lacking such specialized
30 lipids. Examples of sterically stabilized liposomes are those
in which part of the vesicle-forming lipid portion of the
liposome (A) comprises one or more glycolipids, such as
monosialoganglioside G_{M1}, or (B) is derivatized with one or
more hydrophilic polymers, such as a polyethylene glycol (PEG)
35 moiety. While not wishing to be bound by any particular

theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a

5   reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters, 1987, 223*, 42; Wu et al., *Cancer Research, 1993, 53*, 3765). Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al., (*Ann. N.Y. Acad. Sci., 1987, 507*, 64)

10   reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al., (*Proc. Natl. Acad. Sci. U.S.A., 1988, 85*, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both

15   to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are

20   disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn., 1980, 53*, 2778) described liposomes comprising a

25   nonionic detergent, $2C_{12}15G$, that contains a PEG moiety. Illum et al. (*FEBS Lett., 1984, 167*, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups

30   of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett., 1990, 268*, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG

stearate have significant increases in blood circulation half-lives.   Blume et al. (*Biochimica et Biophysica Acta,* **1990,** *1029,* 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of
5 distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher.   Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use
10 thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both
15 to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.).   U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further
20 derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes.   U.S. Patent No. 5,264,221 to Tagawa et al.
25 discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising
30 antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable
35 that they are easily able to penetrate through pores which are

smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g., they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting,

5 and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as

10 effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of

15 the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in

20 *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products

25 and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters,

30 and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of
5 amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the
10 soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The
15 quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include
20 acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p.
25 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of
30 animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a

penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

5   Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in
10   greater detail.

       Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the
15   surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl
20   sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

25   Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-
30   glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (e.g., methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof

(i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**,
5   *7*, 1-33; El Hariri et al., J. Pharm. Pharmacol., **1992**, *44*, 651-654).

     Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's
10 *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as
15 well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate),
20 glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-
25 24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**,
30 pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, **1992**, *263*, 25; Yamashita et al., *J. Pharm. Sci.*, **1990**, *79*, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of

5 oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are

10 thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339).   Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and

15 homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Buur et al., *J. Control Rel.*,

20 **1990**, *14,* 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that

25 nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7,* 1-33).   This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives

30 (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and

phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and
5   other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the
10 cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

15 **Carriers**

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not
20 possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The
25 coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier
30 compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-

disulfonic acid (Miyao et al., *Antisense Res. Dev.*, **1995**, *5,* 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6,* 177-183).

Excipients

5   In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the

10   planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g.,

15   pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium

20   stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate,

25   etc.).
         Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention.

30   Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids
may include sterile and non-sterile aqueous solutions, non-
aqueous solutions in common solvents such as alcohols, or
solutions of the nucleic acids in liquid or solid oil bases.
5 The solutions may also contain buffers, diluents and other
suitable additives. Pharmaceutically acceptable organic or
inorganic   excipients   suitable   for   non-parenteral
administration which do not deleteriously react with nucleic
acids can be used.

10   Suitable pharmaceutically acceptable excipients include,
but are not limited to, water, salt solutions, alcohol,
polyethylene glycols, gelatin, lactose, amylose, magnesium
stearate,   talc,   silicic   acid,   viscous   paraffin,
hydroxymethylcellulose, polyvinylpyrrolidone and the like.
15

Other Components

The compositions of the present invention may
additionally contain other adjunct components conventionally
found in pharmaceutical compositions, at their art-established
20 usage levels. Thus, for example, the compositions may contain
additional, compatible, pharmaceutically-active materials such
as, for example, antipruritics, astringents, local anesthetics
or anti-inflammatory agents, or may contain additional
materials useful in physically formulating various dosage
25 forms of the compositions of the present invention, such as
dyes,   flavoring   agents,   preservatives,   antioxidants,
opacifiers, thickening agents and stabilizers. However, such
materials, when added, should not unduly interfere with the
biological activities of the components of the compositions
30 of the present invention. The formulations can be sterilized
and, if desired, mixed with auxiliary agents, e.g.,
lubricants, preservatives, stabilizers, wetting agents,
emulsifiers, salts for influencing osmotic pressure, buffers,
colorings, flavorings and/or aromatic substances and the like

which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not

limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See,

5 generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

10   In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of

15 antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and

20 responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body

25 of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in in vitro and

30 in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured

35 residence times and concentrations of the drug in bodily

fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging

5   from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate

10   the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

15   2'-Deoxy   and   2'-methoxy   beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g., Chemgenes, Needham, MA, or Glen Research, Inc., Sterling, VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by

20   reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides   containing   5-methyl-2'-deoxycytidine

25   (5-Me-C) nucleotides were synthesized according to published methods (Sanghvi et al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling, VA, or ChemGenes, Needham, MA).

2'-Fluoro amidites

30   **2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described

previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing

5 commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in

10 moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

15 **2'-Fluorodeoxyguanosine**

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-

20 arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then

25 deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-

30 anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard

5   procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are
10 prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

15 5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide
20 gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount
25 of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 hours) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with
30 the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and
5   placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the
10 filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product
15 was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was
20 co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was
25 added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with
30 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et_3NH. The pure

fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 hour using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours.

The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was
5   evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

10   **2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated
15   and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were
20   evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

        N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-
25   **methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be
30   approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and

evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the

5 title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L).

10 Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was

15 extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting

20 solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

25   2'-(Dimethylaminooxyethoxy) nucleoside amidites (also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-

30 methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and

with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0 g, 0.416 mmol), dimethylaminopyridine (0.66 g, 0.013 eq, 0.0054 mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8 g, 119.0 mL, 1.1 eq, 0.458 mmol) was added in one portion. The reaction was stirred for 16 hours at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600 mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1 mm Hg, 24 hours) to 149 g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath

until an internal temperature of 160 °C was reached and then
maintained for 16 hours (pressure < 100 psig). The reaction
vessel was cooled to ambient and opened. TLC (Rf 0.67 for
desired product and Rf 0.82 for ara-T side product, ethyl
5 acetate) indicated about 70% conversion to the product. In
order to avoid additional side product formation, the reaction
was stopped, concentrated under reduced pressure (10 to 1 mm
Hg) in a warm water bath (40-100°C) with the more extreme
conditions   used   to   remove   the   ethylene   glycol.
10 (Alternatively, once the low boiling solvent is gone, the
remaining solution can be partitioned between ethyl acetate
and water. The product will be in the organic phase.) The
residue was purified by column chromatography (2 kg silica
gel, ethyl acetate-hexanes gradient 1:1 to 4:1).   The
15 appropriate fractions were combined, stripped and dried to
product as a white crisp foam (84 g, 50%), contaminated
starting material (17.4 g) and pure reusable starting material
20g. The yield based on starting material less pure recovered
starting material was 58%. TLC and NMR were consistent with
20 99% pure product.

**2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-
5-methyluridine**

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-
methyluridine   (20   g,   36.98   mmol)   was   mixed   with
25 triphenylphosphine   (11.63   g,   44.36   mmol)   and   N-
hydroxyphthalimide (7.24 g, 44.36 mmol). It was then dried
over P_2O_5 under high vacuum for two days at 40°C. The reaction
mixture was flushed with argon and dry THF (369.8 mL, Aldrich,
sure seal bottle) was added to get a clear solution. Diethyl-
30 azodicarboxylate (6.98 mL, 44.36 mmol) was added dropwise to
the reaction mixture. The rate of addition is maintained such
that resulting deep red coloration is just discharged before
adding the next drop. After the addition was complete, the

reaction was stirred for 4 hours. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane

5 (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

10   2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1 g, 4.5 mmol) was dissolved in dry CH_2Cl_2 (4.5 mL) and methylhydrazine (300 mL, 4.64 mmol) was added dropwise at -10°C to 0°C. After 1 hour, the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the

15 combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5 mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was strirred

20 for 1 hour. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-
25   dimethylaminooxyethyl]-5-methyluridine

        5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77 g, 3.12 mmol) was dissolved in a solution of 1 M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6 mL). Sodium

30 cyanoborohydride (0.39 g, 6.13 mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that, the reaction

vessel was removed from the ice bath and stirred at room temperature for 2 hours, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20 mL). Ethyl acetate

5 phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1 M PPTS in MeOH (30.6 mL). Formaldehyde (20% w/w, 30 mL, 3.37 mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath,

10 sodium cyanoborohydride (0.39 g, 6.13 mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hours. To the reaction mixture 5% $NaHCO_3$ (25 mL) solution was added and extracted

15 with ethyl acetate (2x25 mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-

20 methyluridine as a white foam (14.6 g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91 mL, 24.0 mmol) was dissolved in dry THF and triethylamine (1.67 mL, 12 mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then

25 added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40 g, 2.4 mmol) and stirred at room temperature for 24 hours. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and

30 eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766 mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750 mg, 2.17 mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20 mL). The residue obtained was dissolved in pyridine (11 mL) under argon atmosphere. 4-dimethylaminopyridine (26.5 mg, 2.60 mmol), 4,4'-dimethoxytrityl chloride (880 mg, 2.60 mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13 g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08 g, 1.67 mmol) was co-evaporated with toluene (20 mL). To the residue N,N-diisopropylamine tetrazonide (0.29 g, 1.67 mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4 mL) and 2-cyanoethyl-N,N,N[1],N[1]-tetraisopropylphosphoramidite (2.12 mL, 6.08 mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hours under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70 mL) and washed with 5% aqueous $NaHCO_3$ (40 mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-

dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04 g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites (also known in
the art as 2'-O-(aminooxyethyl) nucleoside amidites) are
prepared as described in the following paragraphs. Adenosine,
cytidine and thymidine nucleoside amidites are prepared
similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained
by selective 2'-O-alkylation of diaminopurine riboside.
Multigram quantities of diaminopurine riboside may be
purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount
of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine
riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase.
(McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501
A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to
provide   2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before
the hydroxyl group may be displaced by N-hydroxyphthalimide
via a Mitsunobu reaction, and the protected nucleoside may
phosphitylated   as   usual   to   yield   2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalmidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH$_2$-O-CH$_2$-N(CH$_2$)$_2$, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetra-hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethyl-aminoethoxy)ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylamino-ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl,

0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution

5 and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using $MeOH:CH_2Cl_2:Et_3N$ (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-
10   dimethylaminoethoxy)ethyl)]-5-methyl uridine-3'-O-
(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-
15 dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent
20 to give the title compound.

Example 2
Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA
25 synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-
30 3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step.

After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hours), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate
5 oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

10   3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878,
15 herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

20   3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by
25 reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

30 **Oligonucleoside Synthesis**

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethyl-hydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked

oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for

5 instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides

10 are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

15 **Example 4**

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential

20 Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

25 **Synthesis of Chimeric Oligonucleotides**

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between

30 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound.

Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

5   **[2'-O-Me] -- [2'-deoxy] -- [2'-O-Me]   Chimeric Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated

10 DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by

15 increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized

20 to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched

25 with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

30   **[2'-O-(2-Methoxyethyl)] -- [2'-deoxy] -- [2'-O-(Methoxyethyl)]   Chimeric   Phosphorothioate Oligonucleotides**

[2'-O-(2-methoxyethyl)] -- [2'-deoxy] -- [-2'-O-(methoxy-

ethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

5   **[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy**
           Phosphorothioate]--[2'-O-(2-Methoxyethyl)
           Phosphodiester] Chimeric Oligonucleotides

           [2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phos-
           phorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric
10   oligonucleotides are prepared as per the above procedure for
      the 2'-O-methyl chimeric oligonucleotide with the substitution
      of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites,
      oxidization with iodine to generate the phosphodiester
      internucleotide linkages within the wing portions of the
15   chimeric structures and sulfurization utilizing 3,H-1,2
      benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate
      the phosphorothioate internucleotide linkages for the center
      gap.

           Other chimeric oligonucleotides, chimeric oligonucleo-
20   sides and mixed chimeric oligonucleotides/oligonucleosides are
      synthesized according to U.S. Patent 5,623,065, herein
      incorporated by reference.

      Example 6

      Oligonucleotide Isolation

25   After cleavage from the controlled pore glass column
      (Applied Biosystems) and deblocking in concentrated ammonium
      hydroxide at 55°C for 18 hours, the oligonucleotides or
      oligonucleosides are purified by precipitation twice out of
      0.5 M NaCl with 2.5 volumes ethanol. Synthesized
30   oligonucleotides were analyzed by polyacrylamide gel
      electrophoresis on denaturing gels and judged to be at least
      85% full length material. The relative amounts of

phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol.*

5 *Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

10   Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate

15 internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g., PE-Applied Biosystems, Foster

20 City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

        Oligonucleotides were cleaved from support and

25 deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic

30 pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

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The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

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25

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

30

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies,

Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were

5 routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be

10 seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained

15 from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100

20 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained

25 from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

30 HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville, MD). HEKs were

routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

5 HepG2 cells:

      The human hepatoblastoma cell line HepG2 was obtained from the American Type Culure Collection (Manassas, VA). HepG2 cells were routinely cultured in Eagle's MEM supplemented with 10% fetal calf serum, non-essential amino 10 acids, and 1 mM sodium pyruvate (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.
15   For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:
20   When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μL of OPTI-MEM™-1 containing 3.75 μg/mL LIPOFECTIN™ (Gibco BRL) and the desired 25 concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.
      The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal 30 oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG,**

SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**,

5   SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS

10 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as

15 the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

Example 10

20 **Analysis of oligonucleotide inhibition of hormone-sensitive lipase expression**

        Antisense modulation of hormone-sensitive lipase expression can be assayed in a variety of ways known in the art. For example, hormone-sensitive lipase mRNA levels can

25 be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example,

30 Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current*

Protocols in Molecular Biology, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available

5 from PE-Applied Biosystems, Foster City, CA, and used according to manufacturer's instructions.

Protein levels of hormone-sensitive lipase can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis

10 (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to hormone-sensitive lipase can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation

15 methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al.,

20 *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1- 11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-

25 10.16.11, John Wiley & Sons, Inc., **1998**. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays

30 (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+
5 mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold PBS. 60
10 μL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μL of lysate was transferred to Oligo d(T) coated 96-well plates
15 (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μL of
20 elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be
25 treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following
30 the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold PBS. 100 μL Buffer RLT was added to each well and the plate vigorously

agitated for 20 seconds. 100 μL of 70% ethanol was then added
to each well and the contents mixed by pipetting three times
up and down. The samples were then transferred to the RNEASY
96™ well plate attached to a QIAVAC™ manifold fitted with a
5   waste collection tray and attached to a vacuum source. Vacuum
was applied for 15 seconds. 1 mL of Buffer RW1 was added to
each well of the RNEASY 96™ plate and the vacuum again applied
for 15 seconds. 1 mL of Buffer RPE was then added to each
well of the RNEASY 96™ plate and the vacuum applied for a
10   period of 15 seconds. The Buffer RPE wash was then repeated
and the vacuum was applied for an additional 10 minutes. The
plate was then removed from the QIAVAC™ manifold and blotted
dry on paper towels. The plate was then re-attached to the
QIAVAC™ manifold fitted with a collection tube rack containing
15   1.2 mL collection tubes. RNA was then eluted by pipetting 60
μL water into each well, incubating 1 minute, and then
applying the vacuum for 30 seconds. The elution step was
repeated with an additional 60 μL water.

        The repetitive pipetting and elution steps may be
20   automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc.,
Valencia, CA). Essentially, after lysing of the cells on the
culture plate, the plate is transferred to the robot deck
where the pipetting, DNase treatment and elution steps are
carried out.

25   **Example 13**
**Real-time Quantitative PCR Analysis of hormone-sensitive
lipase mRNA Levels**

        Quantitation of hormone-sensitive lipase mRNA levels was
determined by real-time quantitative PCR using the ABI PRISM™
30   7700 Sequence Detection System (PE-Applied Biosystems, Foster
City, CA) according to manufacturer's instructions. This is
a closed-tube, non-gel-based, fluorescence detection system
which allows high-throughput quantitation of polymerase chain

reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by

5   including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA, or PE-Applied Biosystems,

10 Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA, or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is

15 quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq

20 polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored

25 at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense

30 oligonucleotide treatment of test samples.

        Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the

35 internal standard gene GAPDH are amplified concurrently in a

single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing).

5   Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10%

10 of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems,

15 Foster City, CA. RT-PCR reactions were carried out by adding 25 μL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl$_2$, 300 μM each of dATP, dCTP and dGTP, 600 μM of dUTP, 900 nM of forward primer, 50 nM of reverse primer, and 100 nM of probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units

20 MuLV reverse transcriptase) to 96 well plates containing 25 μL total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15

25 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA

30 using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification

35 by RiboGreen™ are taught in Jones, L.J., et al., *Analytical*

Biochemistry, **1998**, *265*, 368-374.

In this assay, 175 μL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL

5 purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human hormone-sensitive lipase were designed to hybridize to a human hormone-sensitive lipase

10 sequence, using published sequence information (GenBank accession number NM_005357, incorporated herein as SEQ ID NO: 3). For human hormone-sensitive lipase the PCR primers were: forward primer: ACCTGCGCACAATGACACA (SEQ ID NO: 4) reverse primer: TGGCTCGAGAAGAAGGCTATG (SEQ ID NO: 5) and the

15 PCR probe was: FAM-CCTCCGCCAGAGTCACCAGCG-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:

20 forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7) reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied

25 Biosystems, Foster City, CA) is the quencher dye.

Probes and primers to mouse hormone-sensitive lipase were designed to hybridize to a mouse hormone-sensitive lipase sequence, using published sequence information (GenBank accession number U08188, incorporated herein as SEQ ID NO:

30 10). For mouse hormone-sensitive lipase the PCR primers were: forward primer: TGCACCACTGAACTGAGCTG (SEQ ID NO: 11) reverse primer: CCGCCCCACTTACTGTCTC (SEQ ID NO: 12) and the PCR probe was: FAM-CGGCGGGGGGCGGCACTAAAAGACCTCTTGCTCCCATCTGCGCGGGCTTC-TAMRA

35 (SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City,

CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For mouse GAPDH the PCR primers were:

forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO: 14)

5 reverse primer: GGGTCTCGCTCCTGGAAGCT (SEQ ID NO: 15) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC- TAMRA 3' (SEQ ID NO: 16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

10 **Example 14**

Northern blot analysis of hormone-sensitive lipase mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was 15 prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon 20 membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV 25 Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then robed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human hormone-sensitive lipase, a human 30 hormone-sensitive lipase specific probe was prepared by PCR using the forward primer ACCTGCGCACAATGACACA (SEQ ID NO: 4) and the reverse primer TGGCTCGAGAAGAAGGCTATG (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency

membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

To detect mouse hormone-sensitive lipase, a mouse hormone-sensitive lipase specific probe was prepared by PCR using the forward primer TGCACCACTGAACTGAGCTG (SEQ ID NO: 11) and the reverse primer CCGCCCCACTTACTGTCTC (SEQ ID NO: 12). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Design of chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap targeting human hormone-sensitive lipase

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human hormone-sensitive lipase RNA, using published sequences (GenBank accession number NM_005357, incorporated herein as SEQ ID NO: 3, GenBank accession number L11706, incorporated herein as SEQ ID NO: 17 and GenBank accession number AA635891, incorporated herein as SEQ ID NO: 18). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl

(2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide.

TABLE 1

Design of human hormone-sensitive lipase mRNA chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
129365	5'UTR	3	172	TTGATTCCTCATGATGGCAC	19
129366	5'UTR	3	174	CATTGATTCCTCATGATGGC	20
129367	5'UTR	3	185	CACAGATCTCTCATTGATTC	21
129368	5'UTR	3	189	TCTTCACAGATCTCTCATTG	22
129369	5'UTR	3	226	CAGGTTCTATCCTTCTGGGC	23
129370	5'UTR	3	250	CCCTCACGGGAGATATTGAT	24
129371	5'UTR	3	269	CCTGGCTCCATTGTTATTTC	25
129372	Start Codon	3	280	CTGACTTAGAACCTGGCTCC	26
129373	Coding	3	348	TTCTGGCCCAGGCTCTAGCG	27
129374	Coding	3	401	TGGGTATTGGATCCCTGCAG	28
129375	Coding	3	617	CCTAGCCCAGGTCCCTGCTG	29
129376	Coding	3	752	GCTCCAGGTTTAGCCTGGGC	30
129377	Coding	3	929	GCCTTCCACTCTAGGGCTGA	31
129378	Coding	3	951	ATCTGCGACCCACTCAGAAA	32
129379	Coding	3	994	AATCTGTGTCTGAAGATGAT	33
129380	Coding	3	1007	ATCGTGGCTGGAGAATCTGT	34
129381	Coding	3	1143	GGCTGTATCCTGGTAGTGTC	35
129382	Coding	3	1174	TGCGCAGGTCCATGTTGTGG	36
129383	Coding	3	1202	GCCAGAGTCACCAGCGACTG	37
129384	Coding	3	1214	ATGTTGTCCTCCGCCAGAGT	38
129385	Coding	3	1242	CCCAGGACCCTGGCTCGAGA	39
129387	Coding	3	1384	GGCTGCGGTACCCGTTGGCC	40
129389	Coding	3	1403	CAGCGGGCTGTGTGCACTAG	41
129391	Coding	3	1427	TTGTGCAGGAGGTGCGCCAG	42
129394	Coding	3	1439	ACATAGCGGGATTTGTGCAG	43
129396	Coding	3	1451	CGGTTGGAGGCCACATAGCG	44
129398	Coding	3	1506	CAGGTAGGCCTCCAGCTCGG	45
129400	Coding	3	1595	TCGCCCTCAAAGAAGAGTAC	46
129402	Coding	3	1643	TTATGCAGCGTGACATACTC	47
129404	Coding	3	1651	AGCATCCCTTATGCAGCGTG	48
129406	Coding	3	1674	GAAGCCCAGGCAGCGGCCAT	49
129408	Coding	3	1715	GAGATGGTCTGCAGGAATGG	50
129410	Coding	3	1718	ATGGAGATGGTCTGCAGGAA	51
129412	Coding	3	1852	GTGTGATCCGCTCAAACTCA	52
129414	Coding	3	1919	AGAGACGATAGCACTTCCAT	53
129416	Coding	3	2091	ACGCAGGTCATAGGAGATGA	54
129418	Coding	3	2130	CTTTATCAGGCTGCTGAGCT	55
129420	Coding	3	2227	CCACAAAGCCACCGCCGTGG	56
129422	Coding	3	2233	TCTGGGCCACAAAGCCACCG	57
129424	Coding	3	2352	GCACTCCTCCAGCGCACGGG	58
129426	Coding	3	2368	AGCAGTAGGCGAAGAAGCAC	59
129428	Coding	3	2413	TTCGTTCCCCTGTTGAGCCA	60
129430	Coding	3	2450	CAGAGGTTCCCGCCTGCACT	61
129432	Coding	3	2456	GTGAAGCAGAGGTTCCCGCC	62
129434	Coding	3	2466	AAGAGCCACGGTGAAGCAGA	63
129436	Coding	3	2639	TCCTCCGTCTTTGCACCAGC	64
129438	Coding	3	2700	GGCTGTGTCCCGCCGCACCA	65

129441	Coding	3	2765	CCACTTAACTCCAGGAAGGA	66
129443	Coding	3	2780	TTCTGGGACTTGCGCCCACT	67
129445	Coding	3	2835	CAGTGCTGCTTCAGACACAC	68
129447	Coding	3	2879	AGGTTCTTGAGGGAATCCGT	69
129449	Coding	3	3035	TTTTTGGCCTCAGCCTCTTC	70
129452	Coding	3	3041	AGCTCATTTTTGGCCTCAGC	71
129454	Coding	3	3152	ACTATGGGTGAGGAGTAGAG	72
129456	Coding	3	3294	CTGGCCCAGGTTGCGCAGTC	73
129458	Stop Codon	3	3497	ACAGGCTTTTAGTGTCGCCC	74
129460	3'UTR	3	3534	AAGGCATTCATGACGGAGGC	75
129462	3'UTR	3	3536	GGAAGGCATTCATGACGGAG	76
129464	3'UTR	3	3676	GCAGGTCCAGCCGTCTCGGT	77
129466	5'UTR	17	31	GGTCCCATTCTCAGGACCC	78
129468	5'UTR	17	51	AGAAGTCTAAACCTCCAGTT	79
129470	5'UTR	17	232	CCTGGCCTCCTCGAATCCGG	80
129472	5'UTR	17	265	CTATCACCTCTTTGGGACTC	81
129474	5'UTR	17	450	TTCCTCCTCCTTAGACATAA	82
129476	5'UTR	18	29	ACACATTCATTCAGTAAACG	83
129478	5'UTR	18	95	GTCACCCACCGCTCAAGAGA	84
148862	Coding	3	1158	GTGGATGAGCCTTGAGGCTG	85
148863	Coding	3	1164	CATGTTGTGGATGAGCCTTG	86
148864	Coding	3	1193	ACCAGCGACTGTGTCATTGT	87
148865	Coding	3	1222	AGAAGGCTATGTTGTCCTCC	88
148866	Coding	3	1229	CTCGAGAAGAAGGCTATGTT	89
148867	Coding	3	1237	GACCCTGGCTCGAGAAGAAG	90
148868	Coding	3	1343	AAGAGGTGCGCCACACCCAG	91
148869	Coding	3	1357	CTGGGTCCAGGTCAAAGAGG	92
148870	Coding	3	1377	GTACCCGTTGGCCGGTGTCT	93
148871	Coding	3	1392	GTGCACTAGGCTGCGGTACC	94
148872	Coding	3	1501	AGGCCTCCAGCTCGGCCAGG	95
148873	Coding	3	1515	GAGGGCAGCCAGGTAGGCCT	96
148874	Coding	3	1545	GGCGTAGTAGACCAGAGCGC	97
148875	Coding	3	1590	CTCAAAGAAGAGTACCCCCG	98
148876	Coding	3	1631	ACATACTCCCGGAGGAAGTC	99
148877	Coding	3	1658	CCATAGAAGCATCCCTTATG	100
148878	Coding	3	1663	AGCGGCCATAGAAGCATCCC	101
148879	Coding	3	1736	CCGAAGGACACCAGCCCAAT	102
148880	Coding	3	1788	GAGAGAGCTGGCGGCCACAC	103
148881	Coding	3	1805	AAGCGGCCGCTGGTGAAGAG	104
148882	Coding	3	1902	CATCTCGGTGATGTTCCAGA	105
148883	Coding	3	1910	AGCACTTCCATCTCGGTGAT	106
148884	Coding	3	1955	CGGCTTACCCTCACGGTGGC	107
148885	Coding	3	1986	CTCAAAGGCTTCGGGTGGCA	108
148886	Coding	17	1444	GTGGCATCTCAAAGGCTTCG	109
148887	Coding	3	1998	AGTCAGTGGCATCTCAAAGG	110
148888	Coding	3	2070	CCTGACGAGGACGGGCCCAG	111
148889	Coding	3	2099	TGTCCTTCACGCAGGTCATA	112
148890	Coding	3	2140	GGCCGTTGGACTTTATCAGG	113
148891	Coding	3	2152	CCAGGCTCCGTTGGCCGTTG	114
148892	Coding	3	2217	ACCGCCGTGGAAGTGCACTA	115
148893	Coding	3	2273	TGGGCCCAGCTCTTGAGGTA	116
148894	Coding	3	2362	AGGCGAAGAAGCACTCCTCC	117
148895	Coding	3	2373	GGCCCAGCAGTAGGCGAAGA	118
148896	Coding	3	2382	GTGCTTGATGGCCACGCAGT	119
148897	Coding	3	2393	AGGAGGGCGCAGTGCTTGAT	120
148898	Coding	3	2405	CCTGTTGAGCCAAGGAGGGC	121
148899	Coding	17	1928	GCTGCTGCCCGAAGAGCCAC	122
148900	Coding	3	2504	ATGCCATCTGGCACCCGCAC	123
148901	Coding	3	2531	AGCATTGTGGCCGGGTAGGC	124
148902	Coding	3	2541	GGCAGGCTGCAGCATTGTGG	125
148903	Coding	3	2571	CATGAGGCTCAGCAGGCGGG	126
148904	Coding	3	2610	GACACACTTGGAGAGCACAC	127
148905	Coding	3	2634	CGTCTTTGCACCAGCATAGG	128

148906	Coding	3	2646	GGAGTGGTCCTCCGTCTTTG	129
148907	Coding	3	2665	GGGCTTTCTGGTCTGAGTTG	130
148908	Coding	3	2707	GGAGCAGGGCTGTGTCCCGC	131
148909	Coding	3	2717	AAGTCTCGGAGGAGCAGGGC	132
148910	Coding	3	2740	GCCATGAGGAGGCACCCAGG	133
148911	Coding	3	2757	CTCCAGGAAGGAGTTGAGCC	134
148912	Coding	3	2771	TTGCGCCCACTTAACTCCAG	135
148913	Coding	3	2796	TATGGGCTCCGACATCTTCT	136
148914	Coding	3	2805	CGGCTCTGCTATGGGCTCCG	137
148915	Coding	3	2828	GCTTCAGACACACTGCGGCG	138
148916	Coding	3	2899	GGCTCAAGTCCCTCAGGGTC	139
148917	Coding	3	2954	TCAGCTGACAGCGACATCTC	140
148918	Coding	3	2997	TAATAAGAAGTTGACATCGG	141
148919	Coding	3	3017	TCCCCTGCATCCTCAGGTGG	142
148920	Coding	3	3068	ACGCCCAGGCCTCTGTCCAT	143
148921	Coding	3	3121	TGGCACCCTGGCTGGAGCGT	144
148922	Coding	3	3185	GGTGCCAGCAGCGGCGACAT	145
148923	Coding	3	3199	TGAGCATGCTGTCGGGTGCC	146
148924	Coding	3	3222	GATGTGCACAGGTGGCAGGC	147
148925	Coding	3	3304	GCGTCACCGGCTGGCCCAGG	148
148926	Coding	3	3331	CGTGCGGCAGGTCCTCCACC	149
148927	Coding	3	3350	GCCGCTAGGGTCAGGAAGCC	150
148928	Coding	3	3396	GCGCTCCACGCACAGCTCTG	151
148929	3'UTR	3	3516	GCCGGCGCAGATGGGAACAA	152
148930	3'UTR	3	3544	CCCGGCCCGGAAGGCATTCA	153
148931	3'UTR	3	3574	TTAAGTAAGCACAGCCCGCG	154
148932	3'UTR	3	3585	CCACCCCCGACTTAAGTAAG	155
148933	3'UTR	3	3625	GGCGAGGGTCTCAGCTTTCG	156
148934	3'UTR	3	3685	CGGTGGCGTGCAGGTCCAGC	157
148935	3'UTR	3	3756	AAACCGACCTGCAAGGGAGG	158

Example 16

Antisense inhibition of human hormone-sensitive lipase expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a subset of the series of oligonucleotides which were designed to target different regions of the human hormone-sensitive lipase RNA, using published sequences (GenBank accession number NM_005357, incorporated herein as SEQ ID NO: 3, GenBank accession number L11706, incorporated herein as SEQ ID NO: 17 and GenBank accession number AA635891, incorporated herein as SEQ ID NO: 18) were analyzed for their effect on human hormone-sensitive lipase mRNA levels by quantitative real-time PCR as described in other examples herein.

Data are averages from two experiments. If present, "N.D." indicates "no data". The oligonucleotides are shown

in Table 2. "Target site" indicates the first (5'-most)
nucleotide number on the particular target sequence to which
the oligonucleotide binds. All compounds in Table 2 are
chimeric oligonucleotides ("gapmers") 20 nucleotides in
5 length, composed of a central "gap" region consisting of ten
2'-deoxynucleotides, which is flanked on both sides (5' and
3' directions) by five-nucleotide "wings". The wings are
composed of 2'-methoxyethyl (2'-MOE)nucleotides. The
internucleoside (backbone) linkages are phosphorothioate (P=S)
10 throughout the oligonucleotide. All cytidine residues are 5-
methylcytidines.

TABLE 2

Inhibition of human hormone-sensitive lipase mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
129432	Coding	3	2456	GTGAAGCAGAGGTTCCCGCC	72	62
129449	Coding	3	3035	TTTTTGGCCTCAGCCTCTTC	78	70
148865	Coding	3	1222	AGAAGGCTATGTTGTCCTCC	5	88
148876	Coding	3	1631	ACATACTCCCGGAGGAAGTC	50	99
148878	Coding	3	1663	AGCGGCCATAGAAGCATCCC	0	101
148880	Coding	3	1788	GAGAGAGCTGGCGGCCACAC	33	103
148882	Coding	3	1902	CATCTCGGTGATGTTCCAGA	36	105
148884	Coding	3	1955	CGGCTTACCCTCACGGTGGC	78	107
148885	Coding	3	1986	CTCAAAGGCTTCGGGTGGCA	79	108
148888	Coding	3	2070	CCTGACGAGGACGGGCCCAG	64	111
148889	Coding	3	2099	TGTCCTTCACGCAGGTCATA	46	112
148892	Coding	3	2217	ACCGCCGTGGAAGTGCACTA	72	115
148893	Coding	3	2273	TGGGCCCAGCTCTTGAGGTA	15	116
148894	Coding	3	2362	AGGCGAAGAAGCACTCCTCC	44	117
148895	Coding	3	2373	GGCCCAGCAGTAGGCGAAGA	0	118
148898	Coding	3	2405	CCTGTTGAGCCAAGGAGGGC	85	121
148899	Coding	3	1928	GCTGCTGCCCGAAGAGCCAC	0	122
148900	Coding	3	2504	ATGCCATCTGGCACCCGCAC	68	123
148901	Coding	3	2531	AGCATTGTGGCCGGGTAGGC	65	124
148904	Coding	3	2610	GACACACTTGGAGAGCACAC	29	127
148906	Coding	3	2646	GGAGTGGTCCTCCGTCTTTG	4	129
148907	Coding	3	2665	GGGCTTTCTGGTCTGAGTTG	0	130
148908	Coding	3	2707	GGAGCAGGGCTGTGTCCCGC	0	131
148909	Coding	3	2717	AAGTCTCGGAGGAGCAGGGC	60	132
148910	Coding	3	2740	GCCATGAGGAGGCACCCAGG	67	133
148911	Coding	3	2757	CTCCAGGAAGGAGTTGAGCC	0	134
148916	Coding	3	2899	GGCTCAAGTCCCTCAGGGTC	0	139
148919	Coding	3	3017	TCCCCTGCATCCTCAGGTGG	71	142
148923	Coding	3	3199	TGAGCATGCTGTCGGGTGCC	61	146
148929	3'UTR	3	3516	GCCGGCGCAGATGGGAACAA	22	152

148930	3'UTR	3	3544	CCCGGCCCGGAAGGCATTCA	85	153
148934	3'UTR	3	3685	CGGTGGCGTGCAGGTCCAGC	3	157

As shown in Table 2, SEQ ID NOs 62, 70, 99, 107, 108, 111, 112, 115, 117, 121, 123, 124, 132, 133, 142, 146, and 153

5 demonstrated at least 40% inhibition of human hormone-sensitive lipase expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of

10 the present invention.

Example 17

Design of chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap targeting mouse hormone-sensitive lipase

15 In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the mouse hormone-sensitive lipase RNA, using published sequences (GenBank accession number U08188, incorporated herein as SEQ ID NO: 10). The oligonucleotides are shown in

20 Table 3. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 3 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten

25 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide.

TABLE 3

Design of mouse hormone-sensitive lipase mRNA chimeric
phosphorothioate oligonucleotides having 2'-MOE wings and a
deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
126910	5'UTR	10	238	GCTCCTCTTCAGAATTAGAA	159
126911	5'UTR	10	469	ACCAAGTATTCAAACCTAGG	160
126912	5'UTR	10	521	TTTGCTCTGTCAGGCCCAGG	161
126913	Start Codon	10	585	GCGTAAATCCATGCTGTGTG	162
126914	Coding	10	645	CTGGCTTGAGAAGAAGGCCA	163
126915	Coding	10	665	CGTGCTGTCTCTCCTGGGCC	164
126916	Coding	10	700	GTTCCCGAACACCTGCAAAG	165
126917	Coding	10	710	CCCAGTGCCTGTTCCCGAAC	166
126918	Coding	10	755	AAATGGTGTGCCACACCCAA	167
126919	Coding	10	790	GGTATCCGTTGGCTGGTGTC	168
126920	Coding	10	835	GTAGTAGGTGTGCCAGGCAG	169
126921	Coding	10	854	GCCACATAGCGGGATTTGTG	170
126922	Coding	10	890	TGGCTGGCACGGAAGAAGAT	171
126923	Coding	10	900	TGCTAGGTTGTGGCTGGCAC	172
126924	Coding	10	974	ATGGTCAGCAGGCGCTGGGC	173
126925	Coding	10	994	AGAGCACTCCTGGTCGGTTG	174
126926	Coding	10	1093	TGAACTGGAAGCCCAGGCAG	175
126927	Coding	10	1103	ATGGCAGGTGTGAACTGGAA	176
126928	Coding	10	1120	TCTGCAGGAACGGCCGGATG	177
126929	Coding	10	1140	CACCAGCCCGATGGAGAGAG	178
126930	Coding	10	1172	GTCTCGTTGCGTTTGTAGTG	179
126931	Coding	10	1230	TGGGTCTATGGCGAATCGGC	180
126932	Coding	10	1250	AATTCAGCCCCACGCAACTC	181
126933	Coding	10	1274	TCCAGGTTCTGTATGATGCG	182
126934	Coding	10	1295	AAGGCTTTCCAGAAGTGCAC	183
126935	Coding	10	1300	TCCAGAAGGCTTTCCAGAAG	184
126936	Coding	10	1345	ATGCCATGTTGGCCAGAGAC	185
126937	Coding	10	1373	AGCAGGCGGCTTACCCTCAC	186
126938	Coding	10	1405	GTGGCATCTCAAAGGCCTCA	187
126939	Coding	10	1441	GTGAGATGGTAACTGTGAGC	188
126940	Coding	10	1454	TGTGCCAAGGGAGGTGAGAT	189
126941	Coding	10	1464	TGGTCCCGTGTGTGCCAAGG	190
126942	Coding	10	1487	ATGAGCCTGGCTAGCACAGG	191
126943	Coding	10	1499	AGGTCATAGGAGATGAGCCT	192
126944	Coding	10	1544	GATTTTGCCAGGCTGTTGAG	193
126945	Coding	10	1554	TGGGCCCTCAGATTTTGCCA	194
126946	Coding	10	1646	GAGGTCTGTGCCACAAAGCC	195
126947	Coding	10	1680	GGCCCAGTTCTTGAGGTAGG	196
126948	Coding	10	1690	CTAGCTCCTGGGCCCAGTTC	197
126949	Coding	10	1723	CCAGGGAGTAGTCGATGGAG	198
126950	Coding	10	1785	GACAGCCCAGCAGTAGGCAA	199

126951	Coding	10	1795	CACAGTGCTTGACAGCCCAG	200
126952	Coding	10	1832	GCAAGGCATATCCGCTCTCC	201
126953	Coding	10	1886	GCTGCTGCCCGAAGGGACAC	202
126954	Coding	10	1920	TGCCATGATGCCATCTGGCA	203
126955	Coding	10	1925	TAGGCTGCCATGATGCCATC	204
126956	Coding	10	1946	GACTGCAGGGTGGTAACTGG	205
126957	Coding	10	1967	AGACGAGAGGGAGAAGCAGA	206
126958	Coding	10	2003	ACGCTCAGTGGTAGAAGAGG	207
126959	Coding	10	2063	TCTGAGTCAAAATGGTCCTC	208
126960	Coding	10	2073	TGCCTTCTGGTCTGAGTCAA	209
126961	Coding	10	2105	GTGTCTCTCTGCACCAGCCC	210
126962	Coding	10	2129	CGGAGGTCTCTGAGGAACAG	211
126963	Coding	10	2156	GAGTTGAGCCATGAGGAGGC	212
126964	Coding	10	2243	CTCCTGCGCATAGACTCCGT	213
126965	Coding	10	2263	CCAGGGCTGCCTCAGACACA	214
126966	Coding	10	2278	AGCCCTCAGGCTGGGCCAGG	215
126967	Coding	10	2366	ATTGACTGTGACATCTCGGG	216
126968	Coding	10	2376	AAGTGTCTCCATTGACTGTG	217
126969	Coding	10	2438	GCCTCTTCCTGGGAATTCCC	218
126970	Coding	10	2535	GACACCTTGGCTTGAGCGCC	219
126971	Coding	10	2545	GCATGTGGAGGACACCTTGG	220
126972	Coding	10	2575	GGTTCTTGACTATGGGTGAC	221
126973	Coding	10	2595	CAGCAGAGGAGACATGAAGG	222
126974	Coding	10	2687	CGCGCGAACATGACCGAGTC	223
126975	Coding	10	2732	TCTACCACTTTCAGCGTCAC	224
126976	Coding	10	2820	CAGCCGGATGCGCTGCACGC	225
126977	3'UTR	10	2890	AAGAGGTCTTTTAGTGCCGC	226
126978	3'UTR	10	2999	TTACTGTCTCAAGTTAAGCA	227
126979	3'UTR	10	3030	GGTTCAGCTTTTGGCCCCTG	228
126980	3'UTR	10	3093	AAGGCAGTGGTAGAGTGCAG	229
126981	3'UTR	10	3148	TAACTTTTATTTACAAAAAG	230

Example 18

Western blot analysis of hormone-sensitive lipase protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 hours after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to hormone-sensitive lipase is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the

primary antibody species.   Bands are visualized using a
PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale, CA).

Example 19

Effects of antisense inhibition of hormone-sensitive lipase

5 **(ISIS 126930) on blood glucose levels**

     db/db mice are used as a model of Type 2 diabetes.   These
mice are hyperglycemic, obese, hyperlipidemic, and insulin
resistant.   The db/db phenotype is due to a mutation in the
leptin receptor on a C57BLKS background.   However, a mutation
10 in the leptin gene on a different mouse background can produce
obesity without diabetes (ob/ob mice). These mice were used
in the following studies.

     In accordance with the present invention, ISIS 126930
(GTCTCGTTGCGTTTGTAGTG, SEQ ID NO: 179) was investigated in
15 experiments designed to address the role of hormone-sensitive
lipase in glucose metabolism and homeostasis in ob/ob mice.

     ISIS 126930 is completely complementary to sequences in
the coding region of the human and mouse hormone-sensitive
lipase nucleotide sequences incorporated herein as SEQ ID No:
20 3 (starting at nucleotide 1760 of human hormone-sensitive
lipase; Genbank Accession No. NM_005357) and SEQ ID NO: 10
(starting at nucleotide 1172 of mouse hormone-sensitive
lipase; Genbank Accession No. U08188).

     Male ob/ob mice were divided into groups (n=8) with the
25 same average blood glucose levels and treated by
intraperitoneal injection twice a week with saline or ISIS
126930. Ob/ob mice were treated at a dose of 25 mg/kg of ISIS
126930.   Treatment was continued for 5 weeks with blood
glucose levels being measured on day 0, 7, 14, 21, 28 and 35.
30   By day 28 in ob/ob mice treated with ISIS 126930, blood
glucose levels were reduced from a starting level of 300 mg/dL
to 160 mg/dL and remained at this level through week five.
These final levels are within normal range for wild-type mice

(170 mg/dL). The saline treated levels averaged 250 mg/dL throughout the study.

Example 20

Effects of antisense inhibition of hormone-sensitive lipase

5 **(ISIS 126930) on mRNA expression in liver**

Male ob/ob mice were divided into groups (n=8) with the same average blood glucose levels and treated by intraperitoneal injection twice a week with saline or ISIS 126930 as in Example 19. Treatment was continued for 5 weeks

10 after which the mice were sacrificed and tissues collected for mRNA analysis. RNA values were normalized and are expressed as a percentage of saline treated control.

ISIS 126930 successfully reduced hormone-sensitive lipase mRNA levels in the livers of ob/ob mice (60% reduction of

15 hormone-sensitive lipase mRNA).

Example 21

Effects of antisense inhibition of hormone-sensitive lipase

(ISIS 126930) on liver and fat organ weight

Male ob/ob mice were divided into groups (n=8) with the

20 same average blood glucose levels and treated by intraperitoneal injection twice a week with saline or ISIS 126930 as in Example 19. Treatment was continued for 5 weeks. At day 35 mice were sacrificed and final body weights of mouse liver and fat were measured.

25   Treatment of ob/ob mice with ISIS 126930 resulted in a decrease in liver weight compared to saline-treated controls and no change in fat content. Liver weight was reduced from an average of 4.7 grams to 3.5 grams while fat weight remained the same (average 1.8 grams/mouse).

Example 22

Effects of antisense inhibition of hormone-sensitive lipase
(ISIS 126930) on serum insulin levels

Male ob/ob mice were divided into groups (n=8) with the

5   same average blood glucose levels and treated by
intraperitoneal injection twice a week with saline or ISIS
126930 as in Example 19. Treatment was continued for 5 weeks
with serum insulin levels being measured on day 35.

Mice treated with ISIS 126930 showed a decrease in serum

10 insulin levels compared to controls (57 ng/mL for controls
compared to 8 ng/mL for oligonucleotide-treated animals).

Example 23

Effects of antisense inhibition of hormone-sensitive lipase
(ISIS 126930) on serum AST and ALT levels

15 Male ob/ob mice were divided into groups (n=8) with the
same average blood glucose levels and treated by
intraperitoneal injection twice a week with saline or ISIS
126930 as in Example 19. Treatment was continued for 5 weeks
with AST and ALT levels being measured on day 35. Increased

20 levels of the liver enzymes ALT and AST indicate toxicity and
liver damage.

Mice treated with ISIS 126930 showed a decrease in AST
and ALT levels compared to controls (330 IU/L for AST levels
and 520 IU/L for ALT levels in control animals compared to 250

25 IU/L for both levels in oligonucleotide-treated animals).
These results indicate no ongoing toxic effects of the
oligonucleotide treatment.

Example 24

Effects of antisense inhibition of hormone-sensitive lipase

30 (ISIS 126930) on serum cholesterol and triglyceride levels

Male ob/ob mice were divided into groups (n=8) with the
same average blood glucose levels and treated by

intraperitoneal injection twice a week with saline or ISIS 126930 as in Example 19. Treatment was continued for 5 weeks with serum cholesterol and triglyceride levels being measured on day 35.

5   Mice treated with ISIS 126930 showed a decrease in both serum cholesterol (250 mg/dL for control animals and 150 mg/dL for oligonucleotide-treated animals) and triglycerides (140 mg/dL for control animals and 100 mg/dL for oligonucleotide-treated animals) to normal levels.

10   **EXAMPLE 25**

Effects of antisense inhibition of hormone-sensitive lipase (ISIS 126930) in the P-407 murine model of hyperlipidemia

Poloxamer 407 (P-407), an inert block copolymer comprising a hydrophobic core flanked by hydrophilic 15 polyoxyethelene units, has been shown to induce hyperlipidemia in rodents (Palmer, et al., *Atherosclerosis*, **1998**, *136*, 115-123). In the mouse, one injection, intraperitoneally, of P-407 (0.5g/kg) produced hypercholesterolemia that peaked at 24 hours and returned to control levels by 96 hours following 20 treatment (Saltiel, *Proc. Natl. Acad. Sci. U S A*, **2000**, *97*, 535-537).

C57BL/6 mice, a strain reported to be susceptible to hyperlipidemia-induced atherosclerotic plaque formation were used in the following studies to evaluate antisense 25 oligonucleotides as potential lipid lowering compounds.

Female C57BL/6 mice were divided into three matched groups; (1) wild-type control animals; (2) P-407 injected (0.5g/kg every 3 days) animals and (3) animals receiving a high-cholesterol diet. Control animals received no treatment 30 and were maintained on a normal rodent diet. Mice from each group were dosed intraperitoneally every three days, after fasting overnight, with saline or 50 mg/kg ISIS 126930. Five mice/group were sacrificed at days 0, 0.16, 1, 2, 7, 14, 21,

28, 42, 70 and 140 and evaluated for cholesterol and triglyceride levels, liver enzyme levels, serum glucose levels. At day 140 the remaining animals were sacrificed and evaluated for organ weight and mRNA expression of hormone-
5 sensitive lipase.

Example 26

Evaluation of the P-407 murine model of hyperlipidemia-Time course measurements of serum cholesterol, triglycerides, glucose and liver enzyme levels

10   In order to validate the P-407 model of hyperlipidemia, female C57BL/6 mice of the P-407 treatment group receiving a normal diet (described in Example 25) were evaluated for baseline levels of serum cholesterol and triglycerides, glucose and liver enzyme levels over a time course of 140
15 days. Measurements were taken on days 0, 0.16, 1, 2, 7, 14, 21, 28, 42, 70 and 140.

During the course of the study, all measurments were relatively constant with average serum cholesterol levels remaining at 500 mg/dL; triglyceride levels at 1500 mg/dL; AST
20 levels at 200 IU/L and ALT levels at 100 IU/L. Glucose levels averaged 500 mg/dL over the timecourse of the study.

Example 27

Effects of antisense inhibition of hormone-sensitive lipase (ISIS 126930) in the P-407 murine model of hyperlipidemia-
25 **liver and spleen weights**

Female C57BL/6 mice were divided into three matched groups; (1) wild-type control animals; (2) P-407 injected (0.5 g/kg every 3 days) animals and (3) animals receiving a high-cholesterol diet. Control animals received no treatment
30 and were maintained on a normal rodent diet. Mice from each group were dosed intraperitoneally every three days, after fasting overnight, with saline or 50 mg/kg ISIS 126930. Five

mice/group were sacrificed at day 147 and evaluated for changes in spleen and liver weight as a percent of body weight.

Mice in the saline-injected wild-type group had liver
5 weights that were 4 percent of body weight while those animals receiving ISIS 126930 had liver weights that were 5.5 percent of body weight. Spleen weights in this treatment group were 0.4 percent of body weight for saline-injected animals and 0.58 percent of body weight for animals receiving ISIS 126930.
10 Therefore, antisense treatment of control animals had no deleterious effects on liver or spleen as a function of organ weight compared to saline-injected animals.

Mice in the P-407 treatment group receiving saline had liver weights that were 7 percent of body weight while those
15 animals receiving ISIS 126930 had liver weights that were 7.8 percent of body weight. Spleen weights in this treatment group were 0.5 percent of body weight for saline-injected animals and 0.58 percent of body weight for animals receiving ISIS 126930. Therefore, antisense treatment of P-407 treated
20 animals had no deleterious effects on liver or spleen as a function of organ weight compared to saline-injected animals.

Mice in the high cholesterol diet treatment group receiving saline had liver weights that were 13 percent of body weight while those animals receiving ISIS 126930 had
25 liver weights that were comparable at 12 percent of body weight. Spleen weights in this treatment group were 0.58 percent of body weight for saline-injected animals and 0.6 percent of body weight for animals receiving ISIS 126930.

While liver weights were a greater percent of body weight
30 in animals fed high cholesterol diets than in the other treatment groups, antisense treatment was not found to affect liver weight compared to saline treatment. Consequently, these animals had no deleterious effects on liver or spleen as a function of organ weight compared to saline-injected animals.

Example 28

Effects of antisense inhibition of hormone-sensitive lipase
(ISIS 126930) in the P-407 murine model of hyperlipidemia-
mRNA expression in liver

5   Female C57BL/6 mice were divided into three matched
groups; (1) wild-type control animals; (2) P-407 injected
(0.5 g/kg every 3 days) animals and (3) animals receiving a
high-cholesterol diet as in Example 25. Control animals
received no treatment and were maintained on a normal rodent
10   diet. Mice form each group were dosed intraperitoneally every
three days, after fasting overnight, with saline or 50 mg/kg
ISIS 126930. Five mice/group were sacrificed at day 147 and
evaluated for hormone sensitive lipase expression levels in
the liver.
15   In all three treatment groups, expression levels of
hormone sensitive lipase mRNA in the liver were reduced to
below 10 percent of control.

Example 29

Effects of antisense inhibition of hormone-sensitive lipase
20   (ISIS 126930) in the P-407 murine model of hyperlipidemia-
serum cholesterol and triglyceride levels

        Female C57BL/6 mice were divided into three matched
groups; (1) wild-type control animals; (2) P-407 injected
(0.5 g/kg every 3 days) animals and (3) animals receiving a
25   high-cholesterol diet as in Example 25. Control animals
received no treatment and were maintained on a normal rodent
diet. Mice form each group were dosed intraperitoneally every
three days, after fasting overnight, with saline or 50 mg/kg
ISIS 126930. Five mice/group were sacrificed at day 147 and
30   evaluated for serum cholesterol and triglyceride levels.

        In both the wild-type control group and the high-
cholesterol diet, there was no difference in the levels of
serum cholesterol or triglycerides in animals treated with

either saline or ISIS 126930. All animals in the wild-type control group had serum cholesterol levels of 80 mg/dL while all animals in the high-cholesterol group maintained serum cholesterol levels of 400 mg/dL. Serum triglyceride levels

5   of animals in both wild-type and high-cholesterol groups were below 100 mg/dL.

However, in the P-407 model of hyperlipidemia there was a decrease in both serum cholesterol and triglycerides in the antisense-treated animals. Levels of serum cholesterol in

10   this group dropped from 800 mg/dL in saline-treated animals to 600 mg/dL in animals treated with the antisense compound. Levels of triglycerides showed an even more dramatic decrease going from 1800 mg/dL in saline-treated animals to 600 mg/dL in antisense-treated animals.